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Purification and Characterization of a Novel Ceramidase from *Pseudomonas aerugino a**

(Received for publication, February 2, 1998, and in revised form, March 23, 1998)

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We report here a novel type of ceramidase of *Pseudomonas aeruginosa* AN17 isolated from the skin of a patient with atopic dermatitis. The enzyme was purified 83,400-fold with an overall yield of 21.1% from a culture supernatant of strain AN17. After being stained with a silver staining solution, the purified enzyme showed a single protein band, and its molecular mass was estimated to be 70 kDa on SDS-polyacrylamide gel electrophoresis. The enzyme showed quite wide specificity for various ceramides, *i.e.* it hydrolyzed ceramides containing C12:0–C18:0 fatty acids and 7-nitrobenz-2-oxa-1,3-diazole-labeled dodecanoic acid, and not only ceramide containing sphingosine (d18:1) or sphinganine (d18:0) but also phytosphingosine (t18:0) as the long-chain base. However, the enzyme did not hydrolyze galactosylceramide, sulfatide, GM1, or sphingomyelin, and thus was clearly distinguished from a *Pseudomonas* sphingolipid ceramide *N*-deacylase (Ito, M., Kurita, T., and Kita, K. (1995) *J. Biol. Chem.* 270, 24370–24374). This bacterial ceramidase had a pH optimum of 8.0–9.0, an apparent K_m of 139 μM, and a V_{max} of 5.3 μmol/min/mg using *N*-palmitoylsphingosine as the substrate. The enzyme appears to require Ca^{2+} for expression of the activity. Interestingly, the 70-kDa protein catalyzed a reversible reaction in which the *N*-acyl linkage of ceramide was either cleaved or synthesized. Our study demonstrated that ceramidase is widely distributed from bacteria to mammals.

Ceramide is a common biosynthetic precursor of sphingolipids such as acidic and neutral glycosphingolipids (GSLs)[1] and sphingomyelin (SM). Recently, ceramide has emerged as a second messenger in cell differentiation (1, 2) and apoptosis (3). In the epidermis of mammalian skin, ceramide is produced from SM and glucosylceramide by the action of sphingomyelinase and β-glucosidase, respectively, and then secreted into the extracellular space to form a mantle surrounding individual horny (keratinized) cells (4). This extracellular ceramide, arranged in a lamellar structure, may serve as a major component of the permeability barrier and a skin water reservoir (5).

Ceramidase (CDase, EC 3.5.1.23) is an enzyme that hydrolyzes the *N*-acyl linkage between fatty acids and sphingosine bases in ceramides. It was strongly suggested that CDase plays a crucial role not only in the control of cellular ceramide content but also in the regulation of intracellular signal transduction (6–8). Since their discovery by Gatt in rat brain (9, 10), CDases have been found exclusively in mammals (11–13). Recently the gene of acidic CDase from human urine was cloned and expressed in COS-1 cells (14). However, to date, no CDase has been isolated from prokaryotes.

Although the etiologic factors in atopic dermatitis have yet to be fully elucidated, dry and barrier-disrupted skin is a distinctive feature of this disease which could be evoked by a decrease of ceramide in the stratum corneum (15). Recently, the activity of a SM-deacylase capable of hydrolyzing SM to generate sphingosylphosphorylcholine and fatty acid was detected in atopic dermatitis (16). The activity of this enzyme was considered to relate to the decrease of ceramide in atopic dermatitis (16), since the enzyme may decrease the content of SM which appeared to be the main precursor of ceramide in the human skin. However, a SM-deacylase was originally found in bacteria (17), and thus we examined whether lesions of atopic dermatitis were infected with sphingolipid-degrading bacteria. As a result, we have isolated many sphingolipid-degrading bacteria including SM-deacylase producers from the skin of patients with atopic dermatitis. Surprisingly, the most dominant bacteria among isolates were the unknown CDase producers. This report describes the purification and characterization of a novel type of CDase of *Pseudomonas aeruginosa* AN17 isolated from the skin of a patient with atopic dermatitis.

EXPERIMENTAL PROCEDURES

Materials—^{14}C-Labeled fatty acids (stearic acid, palmitic acid, and lauric acid) were purchased from American Radiolabeled Chemicals Inc. *N*-Palmitoylsphingosine, ceramide III, SM, D-sphingosine, and Triton X-100 were purchased from Sigma. A precoated Silica Gel 60 TLC plate was obtained from Merck (Germany). Sep-Pak Plus Silica, Sep-Pak CM, Sep-Pak QMA, and Sep-Pak C18 cartridges, and bicinchoninic acid protein assay kit were purchased from Waters and Pierce, respectively. All other reagents were of the highest purity available. A type strain of *P. aeruginosa* IFO12689 was obtained from the Institute for Fermentation, Osaka (IFO), Japan.

Patient—AN17 was isolated from a 29-year-old female patient with relatively severe atopic dermatitis, who was attending the dermatologic clinic of our university and receiving no topical corticosteroids. The patient was diagnosed according the clinical criteria (18, 19), past history of other atopic disease, high total IgE level (22,100 units/ml), and typical eczematous skin lesions on the face, neck, and extremities.

Isolation and Identification of AN17—CDase-producing bacteria were isolated from desquamated materials of the patient with atopic dermatitis by an enrichment culture method using a synthetic medium A (0.05% NH_4Cl, 0.05% K_2HPO_4, 0.5% NaCl, and 0.05% TDC, pH 7.2), containing 0.05% SM as the sole source of carbon. When appropriate,

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[1] The abbreviations used are: GSL, glycosphingolipid; CDase, ceramidase; FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high performance liquid chromatography; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PAGE, polyacrylamide gel electrophoresis; SCDase, sphingolipid ceramide *N*-deacylase; SM, sphingomyelin; TDC, taurodeoxycholate; TFAc, trifluoroacetyl.

ceramide was used instead of SM at the same concentration. Briefly, a small amount of desquamated material was suspended in 100 μl of synthetic medium A containing 0.05% SM in an Eppendorf tube and incubated at 30 °C for 3 days. After incubation, 5 μl of culture were transferred to fresh medium and incubated at 30 °C for 3 days. This procedure was repeated four or five times, and then SM-utilizing bacteria were isolated using Trypto-Soya agar plates (Nissui Seiyaku Co., Ltd., Japan) containing 0.01% SM. Each strain of isolated bacteria was cultured in synthetic medium A containing 0.05% SM at 30 °C for 3 days, and the supernatant was then subjected to an assay for CDase activity as described below. The identification of strain AN17 was conducted according to the 8th edition of *Bergy's Manual of Determinative Bacteriology* (20). The main characteristics of AN17 were as follows: Gram-negative rod (0.8 × 1.6 μm), motile with polar flagella, optimum growth at 37 °C, growth at 42 °C positive, O-F test oxidative, catalase positive, oxidase positive, denitrification positive, fluorescent pigment production positive, and GC content 66%. AN17 was thus assigned to *P. aeruginosa*. This strain is maintained in a Trypto-Soya agar slant containing 0.01% SM at our laboratory.

Preparation of ^{14}C-Labeled Sphingolipids and C12-NBD-Ceramide—Syntheses of ^{14}C-labeled ceramides and GSLs were conducted by using the reverse hydrolysis (condensation) reaction of sphingolipid ceramide *N*-deacylase (SCDase) as described in Mitsutake *et al.* (21, 22). Interestingly, SCDase efficiently condensed the free fatty acid to sphingosine, although the enzyme hardly hydrolyzed ceramides (21). C12-NBD-ceramide was also synthesized by SCDase.[2] Briefly, *N*-TFAc-aminododecanoic acid, which was prepared from amino-fatty acid by blocking with TFAc, was condensed with sphingosine by SCDase at pH 10. After the reaction, excess amounts of sphingosine and *N*-TFAc-aminododecanoic acid were separated from *N*-TFAc-amino-ceramide by using Sep-Pak C18, Sep-Pak Plus Silica, and Sep-Pak QMA cartridges. The block was removed by CH_3ONa to produce ω-amino-ceramide, which was then purified with Sep-Pak C18 and Sep-Pak Plus Silica cartridges. Purified ω-amino-ceramide was then labeled with NBD fluoride. C12-NBD-ceramide was finally purified with a Sep-Pak Plus Silica cartridge.

CDase Assay—The activity of CDase was measured using C12-NBD-ceramide as a substrate as described below. The reaction mixture contained 550 pmol of C12-NBD-ceramide and an appropriate amount of the enzyme in 20 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% (w/v) Triton X-100 and 2.5 mM $CaCl_2$. Following incubation at 37 °C for 20 min, the reaction was terminated by heating in a boiling water bath for 5 min. The sample was evaporated, dissolved in 30 μl of chloroform/methanol (2/1, v/v), and applied to a TLC plate, which was developed with solvent I (chloroform, methanol, 25% ammonia, 90/20/0.5, v/v). C12-NBD-fatty acid released by the action of the enzyme and the remaining C12-NBD-ceramide were separated on a TLC and then analyzed and quantified with a Shimadzu CS-9300 chromatoscanner (excitation 475 nm, emission 525 nm). One enzyme unit was defined as the amount capable of catalyzing the release of 1 μmol of C12-NBD-fatty acid/min from the C12-NBD-ceramide under the conditions described above. A value of 10^{-3} units of enzyme was expressed as 1 milliunit in this study.

A reverse hydrolysis reaction was measured by the method described below. The mixture containing 100 pmol of [^{14}C]palmitic acid and 500 pmol of sphingosine in 20 μl of 25 mM Tris-HCl, pH 7.5, containing 0.25% (w/v) Triton X-100 and 2.5 mM $CaCl_2$ was incubated at 37 °C for an appropriate time, and the reaction was terminated by heating in a boiling water bath for 5 min. The sample was evaporated, dissolved in 20 μl of chloroform/methanol (2/1, v/v), and applied to a TLC plate, which was developed with solvent I. An imaging analyzer BAS1000 (Fuji Film, Japan) was used for analysis and quantification of the [^{14}C]ceramide produced and unreacted [^{14}C]palmitic acid.

Purification of CDase—Inocula from an agar slant of the strain AN17 were introduced into a 14-ml test tube containing 4 ml of sterilized PY-medium (0.5% polypeptone, 0.1% yeast extract, 0.5% NaCl, pH 7.2) containing 0.01% SM and 0.05% TDC, and incubated at 30 °C for 1 day with vigorous shaking. The culture was then transferred to four cotton-plugged 2,000-ml flasks each containing 500 ml of the same medium and incubated at 30 °C for 2 days with vigorous shaking. The culture fluid was centrifuged at 5,600 × *g* for 20 min, and the supernatant obtained (1,900 ml) was then applied to a DEAE Sepharose FF column (30 × 120 mm; Amesham Pharmacia), previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1% (w/v) Lubrol PX (buffer A), using a BPLC-600FC HPLC system (Yamazen Co., Japan). The enzyme was eluted from the column with a linear gradient of buffer A with increasing concentration of NaCl up to 1 M at a flow rate of 5 ml/min. The active fractions were pooled (36 ml), supplemented with NaCl to 1 M, and applied to a phenyl-Sepharose FF column (15 × 150 mm; Amersham) equilibrated with buffer A containing 1 M NaCl. The proteins were eluted with an increasing Lubrol PX gradient from 0 to 1% in buffer A, using a BPLC-600FC HPLC system at the flow rate of 5 ml/min. The active fractions were pooled (45 ml), and half of this sample was applied to a POROS PI packed column (4.6 × 100 mm; PerSeptive Biosystems, Inc.) equilibrated with buffer A. The enzyme was eluted with an increasing NaCl gradient from 0 to 1 M in buffer A, using the BioCAD system at the flow rate of 5 ml/min. The active fractions were pooled (15 ml) and concentrated using a Millipore Molcut LGC (10,000-cut), and an aliquot of the samples (0.25 ml) was applied to a TSKgel G3000SW$_{XL}$ column (7.8 × 300 mm; TOSOH Co., Japan) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.3% Lubrol PX. The protein was eluted from the column with the same buffer at a flow rate of 0.5 ml/min. The CDase activity was detected in Fr. 27–33 as shown in Fig. 1*C*. This chromatography using a TSKgel G3000SW$_{XL}$ column was found to be repeatable at least 20 times with high recovery.

Protein Assay and SDS-PAGE—Protein content was determined by the bicinchoninic acid method (Pierce) or SDS-PAGE using bovine serum albumin as the standard. SDS-PAGE was carried out according to the method of Laemmli (23). The proteins on SDS-PAGE were visualized by staining with a silver-staining solution (24), and determined with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm (Table I, Fig. 1*C*).

Measurement of Other Enzymes—Exoglycosidases and proteases were assayed with *p*-nitrophenyl glycosides (25) and Azocoll (26), respectively, as the substrate. SCDase and endoglycoceramidase activities were determined using ^{14}C-labeled GM1 (see structure of GM1 in Table II) as the substrate described in Mitsutake *et al.* (22). Sphingomyelinase activity was measured by the method described in Ito and Yamagata (27).

Determination of Digestion Products by HPLC, Gas Chromatography, and FAB-MS—Sphingosine bases were determined with HPLC essentially as described in Merrill *et al.* (28). Briefly, samples were dissolved in 50 μl of methanol and mixed with 50 μl of the *o*-phthalaldehyde reaction buffer (9.9 ml of 3% boric acid, pH 10.5, mixed with 0.1 ml of ethanol containing 5 mg of *o*-phthalaldehyde and 5 μl of β-mercaptoethanol). After incubation at room temperature for 5 min, 0.25 ml of a mixture of methanol and 5 mM potassium phosphate buffer, pH 7.0, (9/1, v/v), was added. Sample was then applied to an Inertosil ODS-3 column (4.6 × 100 mm; GL Science Inc.) which was equilibrated with the same buffer solution. Sphingosine bases were eluted from the column at a flow rate of 2.0 ml/min using HITACHI L-7100 HPLC system and detected with a HITACHI L-7480 fluorescence detector (excitation 340 nm and emission 455 nm). Gas chromatography of fatty acids was conducted with GC-14A gas chromatography (Shimadzu Co., Japan) using a HR-SS-10 column (30 m, Shinwa Chemical Industries, Ltd., Japan) with the temperature programmed from 150 to 220 °C at the rate of 4 °C/min. Before analyses, fatty acid standards or samples were heated at 80 °C for 2 h in 1 ml of 5% anhydrous HCl in methanol for methanolysis. Sphingosine and fatty acids were also analyzed by positive and negative FAB-MS, respectively, using a Jeol JMS LX-2000 mass spectrometer (Jeol Ltd., Japan). Triethanolamine and 3-nitrobenzylalcohol were used as the matrix for the negative and positive ion mode, respectively.

RESULTS

Production and Purification of CDase of AN17 —In this study, we have isolated many CDase-producing bacteria from desquamated materials of patients with atopic dermatitis using a synthetic medium containing SM or ceramide as the sole source of carbon. Among the isolates, the strain AN17, identified as *P. aeruginosa*, was selected for further study owing to its high productivity of CDase. The amount of CDase in the culture supernatant of AN17 was 1–2 milliunits/ml when the strain was cultivated at 30 °C for 2 days in a PY-medium containing SM. It was found that production of the CDase of this strain was induced by addition of ceramide or SM to the medium. Since the strain secreted sphingomyelinase as well, the SM added was hydrolyzed to generate ceramide which may induce the CDase production of this bacterium.

[2] M. Tani, K. Kita, H. Komori, T. Nakagawa, and M. Ito, unpublished results.

TABLE I
Purification of CDase from P. aeruginosa

Step	Total activity	Total protein	Specific activity	Recovery	Purification
	milliunits	*mg*	*milliunits/mg*	%	*-fold*
Culture fluid	3960	1770	2.23	100	1
DEAE-Sepharose FF	702	64.4	10.9	17.7	4.89
Phenyl-Sepharose FF	1413	8.96	158	35.7	70.9
POROS PI	1123	3.61	311	28.4	139
TSKgel G3000SW$_{XL}$	835	4.48×10^{-3}[a]	186,000	21.1	83,400

[a] Estimated by SDS-PAGE as described in the text.

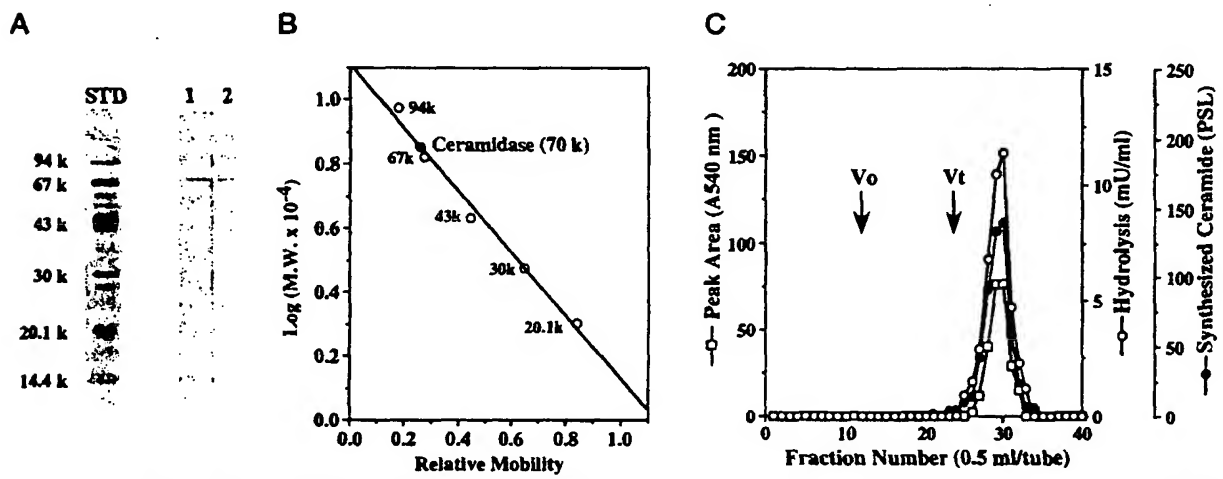

FIG. 1. **Purity and molecular weight of the bacterial CDase.** *A*, the final preparation of CDase was analyzed by SDS-PAGE in the presence (*lane 1*) or absence (*lane 2*) of dithiothreitol. Proteins were stained with a silver-staining solution. *B*, standard proteins (molecular weight in parentheses) are: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100). *C*, gel filtration chromatography using a TSKgel G3000SW$_{XL}$ column was performed as described under "Experimental Procedures." 200 μl of each fraction were analyzed by SDS-PAGE. Proteins were stained with a silver-staining solution, and content of the 70-kDa protein was determined using a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm. Both hydrolysis and condensation activities were determined by the method described under "Experimental Procedures." ○, activity for hydrolysis; ●, activity for condensation; □, elution of the 70-kDa protein (OD at 540 nm); V_o, void volume determined by blue dextran; V_t, bed volume determined by NaCl; *PSL*, photostimulated luminescence.

CDase was purified from 2 liters of culture supernatant of strain AN17 by sequential chromatographies on DEAE Sepharose FF, phenyl-Sepharose FF, POROS PI and TSKgel G3000SW$_{XL}$ as described under "Experimental Procedures." As shown in Table I, CDase was finally purified 83,400-fold with an overall yield of 21.1%. The most effective purification procedure for the bacterial CDase was the gel filtration chromatography using a TSKgel G3000SW$_{XL}$ column, in which the CDase was weakly adsorbed and eluted at about 1.5 bed volumes of the column. Using this chromatography, almost all remaining proteins were separated from the CDase, thereby increasing the purification about 600-fold (Table I). This chromatography seems to work as a kind of adsorption chromatography in which the enzyme may interact with the silica-based gel matrix. However, it should be noted that the chromatography using the TSKgel G3000SW$_{XL}$ column was specifically useful for bacterial alkaline CDase but not for other CDases, such as CDases of mouse liver (data not shown). This result may indicate that the bacterial CDase is somewhat different from other CDases in physical properties. The yield of CDase decreased with the progress of purification, but after phenyl-Sepharose chromatography it was found to increase (Table I), suggesting that some inhibitor(s) for CDase are removed at this step.

Purity and Molecular Mass of Bacterial CDase—The final preparation of bacterial CDase was completely free from the following enzymes: α- and β-galactosidases, α- and β-glucosidases, α- and β-mannosidases, β-*N*-acetylhexosaminidase, α-*N*-acetylgalactosaminidase, α-*N*-acetylglucosaminidase, α-L-fucosidase, proteases, sphingomyelinase, endoglycoceramidase, and SCDase, as was confirmed by activity determination made using 0.1 milliunit of CDase for each assay and incubation with an appropriate substrate for 16 h. The purified enzyme gave a single protein band on SDS-PAGE after staining with a silver-staining solution under both reduced (Fig. 1*A*, *land 1*) and nonreduced conditions (Fig. 1*A*, *lane 2*). The apparent molecular mass of CDase was estimated to be 70 kDa on SDS-PAGE under both conditions (Fig. 1*B*), indicating no subunit is linked by disulfide bond in the enzyme molecule. This contrasts completely with human urine acid CDase (13), which is composed of two subunits of molecular mass of 13 kDa (α) and 40 kDa (β). The 70-kDa protein was most likely to be a CDase, since the elution profile of CDase activity on the chromatography using a TSKgel G3000SW$_{XL}$ column at the final purification step coincided exactly with that of 70-kDa band (Fig. 1*C*) and no other co-eluted bands were observed.

Properties of Bacterial CDase—The optimal activity of the CDase was found around pH 8.5 using 25 mM Tris-HCl buffer (Fig. 2*A*), indicating that this enzyme should be classified as a type of alkaline CDase. The enzyme was potently inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, but not by Mn^{2+} or Mg^{2+} at 1 mM. EGTA and EDTA completely abolished the activity at the same concentration (Fig. 2*B*). After EDTA treatment, Ca^{2+} addition reestablished the enzyme activity at 150% of that before treatment, indicating that Ca^{2+} is the preferred divalent cation. Mn^{2+} addition is about half as effective as Ca^{2+} in reestablish-

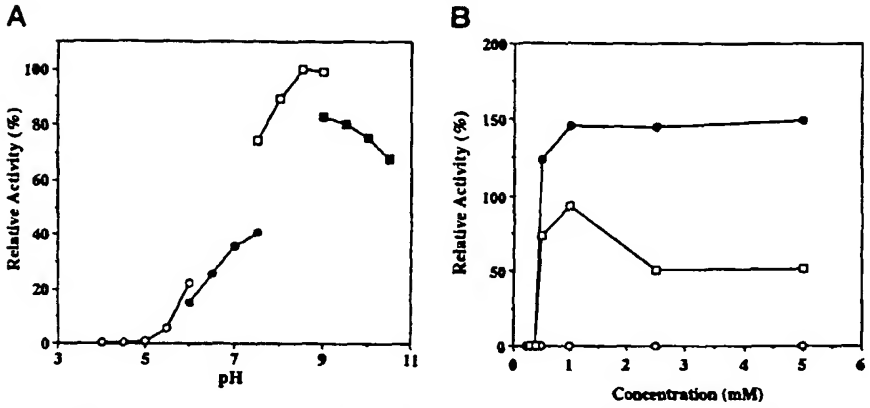

FIG. 2. **pH- and Ca^{2+}-dependence of the bacterial CDase.** *A*, CDase activity was assayed using [^{14}C]ceramide (C16:0, d18:1) as the substrate. [^{14}C]Ceramide was incubated at 37 °C for 2 h with the enzyme in 20 μl of various 25 mM buffers containing 0.25% (w/v) Triton X-100. The ^{14}C-labeled fatty acid released and [^{14}C]ceramide remaining were separated by TLC and analyzed with an imaging analyzer. ○, acetate buffer, pH 4.0–6.0; ●, phosphate buffer, pH 6.0–7.5; □, Tris-HCl buffer, pH 7.5–9.0; ■, glycine-NaOH buffer, pH 9.0–10.5. *B*, 10 μl of mixtures containing 5 microunits of CDase, 1 mM EDTA, and 0.25% (w/v) Triton X-100 in 25 mM Tris-HCl, pH 8.5, were maintained at 5 °C for 30 min. Then, 10 μl of each metal chloride solution at different concentrations in 25 mM Tris-HCl, pH 8.5, containing 100 pmol of C12-NBD-ceramide and 0.25% (w/v) Triton X-100 was added and then incubated at 37 °C for 20 min. The hydrolysis of C12-NBD-ceramide was determined by the method described under "Experimental Procedures." ○, $MgCl_2$; ●, $CaCl_2$; □, $MnCl_2$.

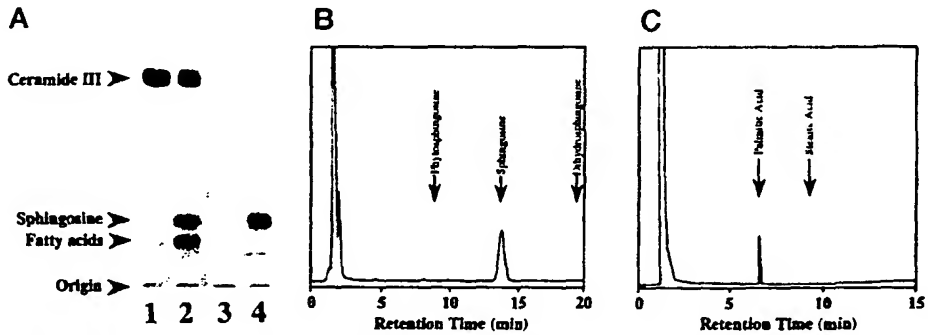

FIG. 3. **Identification of digestion products of ceramides by the enzyme.** *A*, TLC showing digestion products of ceramide III released by the action of enzyme. *Lanes 1* and *3*, ceramide III + boiled enzyme; *lanes 2* and *4*, ceramide III + CDase. *Lanes 1* and *2*, Coomassie Brilliant Blue staining; *lanes 3* and *4*, ninhydrin staining. Ceramide III (10 μg) was incubated at 37 °C for 16 h either with 1 milliunit of CDase or boiled enzyme (100 °C for 5 min) in 200 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% TDC and 2.5 mM $CaCl_2$. After incubation, sample was partitioned with 800 μl of chloroform/methanol (2/1, v/v), and the lower phase was dried, redissolved in 20 μl of chloroform/methanol (2:1, v/v), and applied to a TLC plate which was developed with solvent I. *B*, HPLC showing the sphingosine released from ceramide by the action of enzyme. *N*-Palmitoylsphingosine (20 nmol) was incubated at 37 °C for 16 h with 200 microunits of CDase in 200 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% (w/v) Triton X-100 and 2.5 mM $CaCl_2$. After incubation, sample was partitioned with 800 μl of chloroform/methanol (2/1, v/v), and the lower phase was dried and analyzed by HPLC as described under "Experimental Procedures." *C*, gas chromatography showing the fatty acid released from ceramide by the action of enzyme. Forty nmol of *N*-palmitoylsphingosine were incubated at 37 °C for 16 h with 2 milliunits of CDase in 200 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% (w/v) Triton X-100 and 2.5 mM $CaCl_2$. After incubation, 3 ml of alkaline Dole's solution (isopropyl alcohol, heptane, 1 N NaOH, 40/10/1, v/v) was added and mixed, and then 1.8 ml of heptane and 1.6 ml of water were added. The lower phase obtained after centrifugation was washed twice with 2 ml of heptane. One ml of 1 N H_2SO_4 and 2.4 ml of heptane were added, shaken, and then centrifuged. The upper phase obtained was dried under N_2 gas. Gas chromatography of fatty acids was performed after methanolysis by the method described under "Experimental Procedures."

ing enzyme activity (Fig. 2*B*). It was found that sphingosine (d18:1), sphinganine (d18:0), phytosphingosine (t18:0), and *N*-oleoylethanolamine inhibited enzyme activity by 92.7, 84.2, 39.8, and 67.8%, respectively, at a concentration of 100 μM. The addition of Triton X-100 at a concentration of 0.25–0.5% (w/v) increased the enzyme activity about 6-fold in comparison with that in the absence of the detergent, while the enzyme activity was little affected by TDC or cholate at a concentration up to 1% (w/v). The apparent K_m and the V_{max} were estimated to be 139 μM and 5.3 μmol/min/mg, respectively, using *N*-palmitoylsphingosine as a substrate in a 25 mM Tris-HCl, pH 8.5, containing 0.25% (w/v) Triton X-100. The purified CDase was stable at room temperature (24 °C) for 12 h, but 25 and 100% of the activity were lost after being kept at 37 °C for 12 h and at 60 °C for 5 min, respectively. The enzyme can be kept at −85 °C for at least 2 months without detectable loss of activity in the presence of bovine serum albumin at a final concentration of 1 mg/ml.

Action Mode of Bacterial CDase—Ceramide from bovine brain (ceramide III, Sigma) was digested with the enzyme and digestion products were subjected to TLC which was visualized either with a ninhydrin or a Coomassie Brilliant Blue reagent. A spot having the same R_F of the sphingosine standard (d18:1) was observed on TLC after enzyme treatment (Fig. 3*A*, *lanes 2* and *4*). Fatty acids were also detected when TLC was visualized with a Coomassie reagent (Fig. 3*A*, *lane 2*). Fig. 3*B* shows the chromatogram of HPLC showing sphingosine base released from the authentic ceramide, *N*-palmitoylsphingosine (C16:0, d18:1), by the enzyme. The product showed the same retention time as sphingosine (d18:1). The fatty acid released from the

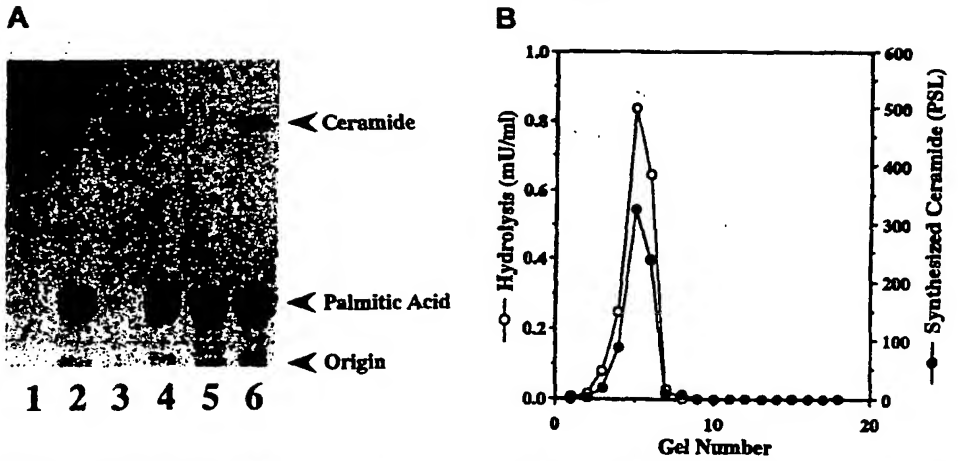

FIG. 4. **Action mode of the bacterial CDase.** *A*, TLC showing the hydrolysis and reverse hydrolysis (condensation) reactions catalyzed by the CDase. *Lane 1*, [^{14}C]ceramide (C16:0, d18:1) standard; *lane 2*, [^{14}C]palmitic acid standard; *lane 3*, [^{14}C]ceramide + boiled enzyme; *lane 4*, [^{14}C]ceramide + CDase; *lane 5*, [^{14}C]palmitic acid + sphingosine + boiled enzyme; *lane 6*, [^{14}C]palmitic acid + sphingosine + CDase. *Lanes 3* and *4*, 100 pmol of [^{14}C]ceramide was incubated with boiled enzyme or 10 microunits of CDase; *lanes 5* and *6*, 200 pmol of sphingosine was incubated at 37 °C for 9 h in 20 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% Triton X-100 with 100 pmol of [^{14}C]palmitic acid in the presence of boiled enzyme (*lane 5*) or 10 microunits of CDase (*lane 6*). After incubation, the sample was applied to a TLC plate, which was developed with solvent I and then analyzed with an imaging analyzer. *B*, detection of hydrolysis and condensation activities of the CDase on Triton-PAGE. 1.5 milliunits of enzyme were applied to a 10% polyacrylamide slab gel containing 0.1% Triton X-100 instead of SDS. The gel was cut into 3-mm slices and crushed in an Eppendorf tube containing 400 μl of 5 mM Tris-HCl, pH 8.5, 0.05% (w/v) Triton X-100, and 0.5 mM $CaCl_2$. After centrifugation at 15,000 rpm for 5 min, 200 μl of supernatant were dried by a Speed Vac concentrator and dissolved in 50 μl of deionized water, and an aliquot was used for the determination of both hydrolysis and condensation activities by the methods described under "Experimental Procedures." ○, hydrolysis; ●, condensation; *PSL*, photostimulated luminescence.

TABLE II
Specificity of CDase from P. aeruginosa

^{14}C-Labeled sphingolipids (100 pmol each) were incubated at 37 °C for 2 h and 12 h (for ceramides) or 16 h (for GSLs and sphingomyelin) with 10 microunits of the enzyme in 20 μl of 25 mM Tris-HCl, pH 8.5, containing 0.25% (w/v) Triton X-100. ^{14}C-Labeled fatty acid and ^{14}C-labeled substrates were separated by TLC and analyzed with an imaging analyzer as described in the text. The extent of hydrolysis (%) was calculated as follows: ^{14}C-labeled fatty acid released × 100/^{14}C-labeled substrate remaining + ^{14}C-labeled fatty acid released.

Substrate	Structure (fatty acid/long-chain base)	Hydrolysis		
		2 h	12 h	16 h
			%	
N-Lauroylsphingosine	C12:0/d18:1	17.6	58.0	
N-Palmitoylsphingosine	C16:0/d18:1	36.7	77.9	
N-Stearoylsphingosine	C18:0/d18:1	26.9	65.3	
N-Palmitoylsphinganine	C16:0/d18:0	23.6	73.9	
N-Stearoylsphinganine	C18:0/d18:0	15.7	62.3	
N-Palmitoylphytosphingosine	C16:0/t18:0	2.0	7.5	
N-Stearoylphytosphingosine	C18:0/t18:0	1.0	4.4	
NBD-*N*-Lauroylsphingosine	NBD-C12:0/d18:1	90.2	100	
GalCer[a]	Galβ1–1'Cer			0
Sulfatide	HSO_3-3Galβ1–1'Cer			0
GM1a	Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–1'Cer			0
Sphingomyelin	Choline phosphate Cer			0

[a] Cer, ceramide.

ceramide by the enzyme was identified to be palmitic acid (C16:0) by using gas chromatography (Fig. 3*C*). Furthermore, the digestion products of the ceramide showed molecular ion peaks at *m/z* 255 and 301, by FAB-MS with negative and positive ion modes, respectively. These peaks coincided with $(M - H)^-$ of palmitic acid (C16:0) and $(M + H)^+$ of sphingosine (d18:1), respectively. These results clearly demonstrate that the enzyme cleaves the *N*-acyl linkage of ceramide to produce sphingosine base and fatty acid.

We found that the bacterial CDase catalyzed not only the hydrolysis reaction but also its reverse reaction. When [^{14}C]ceramide (C16:0, d18:1) was used as a substrate, [^{14}C]palmitic acid was generated by the hydrolysis reaction with the enzyme (Fig. 4*A*, *lane 4*). On the other hand, [^{14}C]ceramide was found to be synthesized when sphingosine was incubated with [^{14}C]palmitic acid in the presence of the enzyme (Fig. 4*A*, *lane 6*). The condensation reaction seems to be due to the action of the enzyme, since boiling of the enzyme at 100 °C for 5 min completely abolished the reaction (Fig. 4*A*, *lane 5*). To determine whether a single protein catalyzed both hydrolysis and condensation reactions, the 70-kDa protein was subjected to Triton-PAGE in which 0.1% Triton X-100 was used instead of SDS. The gel was sliced into 3-mm pieces after electrophoresis, and the enzyme was extracted, before measuring the activities for hydrolysis and condensation reactions. As shown in Fig. 4*B*, the activity for hydrolysis corresponded exactly with that for condensation. Additionally, the elution profile of the 70-kDa protein on the gel filtration chromatography using a TSKgel $G3000SW_{XL}$ column completely coincided with the activities of both hydrolysis and condensation reactions (Fig. 1*C*). These results showed that the 70-kDa protein catalyzed a reversible reaction in which the *N*-acyl linkage of ceramide was cleaved or

synthesized.

Specificity of Bacterial CDase—The substrate specificity of CDase of AN17 was examined using various ^{14}C-labeled sphingolipids. As shown in Table II, CDase hydrolyzed various species of ceramides but not GalCer, sulfatide, GM1, or SM. This result clearly shows that this bacterial CDase is completely different from the SCDase capable of hydrolyzing the *N*-acyl linkage between fatty acids and sphingosine bases in ceramides of various GSLs and SM (17). In contrast to CDase, SCDase hardly attacked the *N*-acyl linkage of ceramides (17). Ceramide containing sphingosine (d18:1), dihydrosphingosine (sphinganine, d18:0), or phytosphingosine (t18:0) was hydrolyzed by the bacterial CDase, but ceramide containing phytosphingosine was more resistant to hydrolysis by the enzyme (Table II). Interestingly, C12-NBD-ceramide (NBD-C12:0, d18:1) was hydrolyzed much faster than *N*-lauroylsphingosine (C12:0, d18:1), suggesting that the susceptibility of ceramide to an alkaline CDase increases with the attachment of NBD to the fatty acid moiety of ceramide.

DISCUSSION

The enzyme of *P. aeruginosa* strain AN17 seems to be a novel type of CDase, judging from the following observations. 1) The 70-kDa protein was the first purified CDase of prokaryotes. Previous reports on CDases were restricted to those from mammalian sources (11–13). 2) Mammalian CDases are classified mainly into two types according to their pH optima: an acidic CDase derived from lysosomes (13) and a membrane-bound alkaline enzyme (6, 7, 12). Since the bacterial CDase shows a pH optimum of 8.0–9.0, it should be classified as an alkaline enzyme. However, the enzyme was a secreted protein and not membrane-bound. 3) The molecular weight and secondary structure of bacterial CDase were clearly different from those of mammals (12, 13). The substrate specificity of the CDase of bacterial origin was not fully compared with that from other sources, because no detailed data for the purified enzyme is available at present except for the bacterial CDase. 4) The bacterial CDase is completely different from SCDase (17), since the CDase did not hydrolyze GSLs or SM. 5) The enzyme was activated by Ca^{2+} and inactivated by EDTA and EGTA. The activity of EDTA-inactivated enzyme was completely restored by addition of Ca^{2+}, suggesting that Ca^{2+} affects the catalytic domain of the enzyme. All CDases reported so far were independent on metal ions including Ca^{2+}.

It was suggested that an acidic ceramidase of rat brain participates in the synthesis as well as hydrolysis of ceramide (9, 10). However, the question of whether a single protein can catalyze both hydrolysis and synthesis reactions, or some other factors are required for ceramide synthesis by CDase remains open, because the enzyme has not yet been purified (9–11). In the present study, we clearly demonstrated that the 70-kDa protein can catalyze the reversible reactions in which the *N*-amide bond of ceramide was either cleaved or synthesized without the assistance of co-factors (Figs. 1*C* and 4).

In the skin of atopic dermatitis, decrease of ceramide content in the stratum corneum (15) and replacement of abnormal fatty acids in the ceramide moiety have been reported (29). These abnormalities might form the dry and barrier-disrupted skin. It was speculated by the present study that bacterial alkaline CDase could contribute directly or indirectly to the abnormality, since the enzyme was found to hydrolyze ceramides isolated from the desquamated materials of patients with atopic dermatitis (data not shown). The surface pH of normal skin is usually neutral or slightly acidic, whereas that of patients with atopic dermatitis tends to alkaline (30), which might suit the alkaline CDase of bacteria.

One significant finding in this study is that the CDase-producing bacterium AN17 was identified to be *P. aeruginosa*, which is a well known opportunistic pathogen (31). It is important to note that the strain *P. aeruginosa* IFO 12689 also retained the ability to produce CDase (data not shown). The role of the CDase as an etiologic factor in atopic dermatitis as well as infectious diseases caused by *P. aeruginosa* should be studied in the future.

Some bacterial sphingolipid-degrading enzymes have proved to be a useful tool for sphingolipid research (17, 27, 32, 33). This study shows it is possible to provide highly purified CDase in a large quantity which will facilitate the study of the structure and function of ceramide.

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